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Monoclonal antibody identification of subpopulations of cerebral cortical neurons affected in Alzheimer disease

(pyramidal neurons/neurofibrillary tangles/immunoblot/immunocytochemistry/disease progression)

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ABSTRACT **Neuronal degeneration is one of the hallmarks of Alzheimer disease (AD). Given the paucity of molecular markers available for the identification of neuronal subtypes, the specificity of neuronal loss within the cerebral cortex has been difficult to evaluate. With a panel of four monoclonal antibodies (mAbs) applied to central nervous system tissues from AD patients, we have immunocytochemically identified a population of vulnerable cortical neurons; a subpopulation of pyramidal neurons is recognized by mAbs 3F12 and 44.1 in the hippocampus and neocortex, and clusters of multipolar neurons in the entorhinal cortex reactive with mAb 44.1 show selective degeneration. Closely adjacent stellate-like neurons in these regions, identified by mAb 6A2, show striking preservation in AD. The neurons recognized by mAbs 3F12 and 44.1, to the best of our knowledge, do not comprise a single known neurotransmitter system. mAb 3A4 identifies a phosphorylated antigen that is undetectable in normal brain but accumulates early in the course of AD in somas of vulnerable neurons. Antigen 3A4 is distinct from material reactive with thioflavin S or antibody generated against paired helical filaments. Initially, antigen 3A4 is localized to neurons in the entorhinal cortex and subiculum, later in the association neocortex, and, ultimately in cases of long duration, in primary sensory cortical regions. mAb 3F12 recognizes multiple bands on immunoblots of homogenates of normal and AD cortical tissues, whereas mAb 3A4 does not bind to immunoblots containing neurofilament proteins or brain homogenates from AD patients. Ultrastructurally, antigen 3A4 is localized to paired-helical filaments. Using these mAbs, further molecular characterization of the affected cortical neurons is now possible.**

Alzheimer disease (AD) is a dementing degenerative disease of the central nervous system (CNS) of unknown cause. Neuronal loss, neurofibrillary tangles, neuritic plaques, amyloid angiopathy, and, less commonly, granulovacuolar degeneration and Hirano bodies constitute the major histopathology.

Whereas much attention has been focused on the neurofibrillary pathology, neuronal loss has been more difficult to evaluate. There are relatively few selective histologic or molecular markers for human neuronal subpopulations, thus limiting identification of the vulnerable neurons. Enzymes important in the biosynthesis of neurotransmitters, such as acetylcholine in the nucleus basalis of Meynert (1) and norepinephrine in the locus ceruleus (2), have been useful correlates in the histochemical analyses of subcortical nuclei in normal and AD tissue. Neuronal loss within the cerebral cortex also may be highly selective. Neuritic plaques and neurofibrillary tangles have been quantified according to their regional distribution and correlated with neuronal loss within the hippocampus and limbic system and in neocortical association areas (3). Phosphorylated and nonphosphorylated neurofilament protein (4, 5), the microtubule-associated proteins tau (6) and microtubule-associated protein 2 (6), and ubiquitin (7) have also been shown to accumulate abnormally in certain tangle-bearing cortical neurons in AD. However, neurofibrillary tangles and neuritic plaques may also occur in nondemented, aged patients. Furthermore, we have reported (8) loss of some retinal ganglion cells and an associated optic neuropathy in some patients with AD. In these sites, no neurofibrillary tangles, plaques, or amyloid angiopathy were detected.

Using immunoperoxidase localization, we have defined (9) a panel of monoclonal antibodies (mAbs) that bind to distinct neuronal subpopulations in the normal human CNS. Three mAbs, 3F12, 44.1, and 6A2, identify hippocampal and neocortical neurons in regions of normal brains that are known to show histopathology in AD. mAb 3F12 binds to a subgroup of large pyramidal neurons, mAb 44.1 binds to a larger population of pyramidal and nonpyramidal neurons, and mAb 6A2 binds to nonpyramidal, stellate-like neurons. In the hippocampal formation, some of the neuronal subpopulations identified by our mAbs were anatomically well segregated, whereas in the neocortex, they were intermixed.

In this study, we have utilized these three mAbs to identify neurons vulnerable to the degenerative process. We have examined the distribution of mAb reactivity in CNS tissues obtained at autopsy from AD patients and from age-matched, nondemented control individuals and patients with other neurological diseases. A fourth mAb (mAb 3A4) recognizes an antigen that is readily detectable in the AD cerebrum but only rarely seen in age-matched controls. The relationship of the antigen expression to the duration of the disease is also examined. Preliminary molecular data on two antigens (antigens 3F12 and 3A4) and the relationship of antigen 3A4 to normal neurofilament proteins and neurofibrillary tangles in AD are presented.

MATERIALS AND METHODS

The preparation and specificities of mAbs 3A4, 3F12, 6A2, and 44.1 have been described (9–11). All four mAbs were of the IgM class.

Tissue Preparation. CNS tissues were obtained postmortem from seven neurologically normal, control patients (ages 25 to 74, with three subjects whose age was more than 60),

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Abbreviations: mAb, monoclonal antibody; CNS, central nervous system; AD, Alzheimer disease; PHF, paired helical filaments.

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one patient with chronic alcoholic dementia (age 76), three patients with amyotrophic lateral sclerosis (ages 38, 60, and 70), three patients with Huntington disease (ages 55, 63, and 65), and nine patients with clinical histories of dementia and histologic diagnoses of AD (12) (ages 72–94). The mean postmortem interval was 3.5 hr (range of 2 to 6 hr) for AD, 7.5 hr (range of 2 to 12 hr) for normal controls, and 10 hr (range of 4 to 19 hr) for the neurologically diseased controls. From all patients, tissue blocks ($\approx$1 cm^3) were dissected from the hippocampus (at the level of the lateral geniculate nucleus), basal ganglia, dorsal root ganglia, and spinal cord. Neocortical sites included superior frontal cortex (Brodmann's area 9), primary visual cortex (area 17), and superior temporal gyrus (area 22). Tissues were rapidly frozen in liquid nitrogen-cooled isopentane and stored at −90°C.

Histology. Four- to 8-μm-thick cryostat sections were processed by the single- or double-labeled immunoperoxidase or immunofluorescence technique. Thioflavin S staining of frozen sections was performed as described by Morrison *et al.* (13). Bielschowsky, Congo red, and Nissl stains were performed according to standard procedures. Phosphatase digestion of frozen tissue sections or nitrocellulose strips from immunoblots used calf intestinal mucosa alkaline phosphatase (Sigma) (50 μg/ml) incubated for 1 hr at 37°C.

Immunoelectron Microscopy. Tissue blocks (1 mm^3) were dissected from stratum pyramidalis of formalin-fixed AD hippocampus, embedded in LR-white resin, thin-sectioned, and stained on the grid. Sections were incubated in 1% normal mouse serum to block nonspecific binding, incubated with undiluted mAb 3A4 hybridoma supernatant overnight at 4°C, washed, and then incubated with 10-nm gold-labeled goat anti-mouse immunoglobulin (Janssen Pharmaceutica, Beerse, Belgium). Sections were then stained with uranyl acetate and viewed with a Zeiss EM10 electron microscope at 60 kV.

Immunoblots. The apparent molecular weights of antigens recognized by the mAbs were determined after electrophoresis on 8% $NaDodSO_4$/polyacrylamide gel and electroblotting onto nitrocellulose sheets (14). Immunoreactive bands were detected by autoradiography after labeling with ^{125}I-conjugated goat anti-mouse antibody or by the ABC-alkaline phosphatase procedure (Vector Laboratories, Burlingame, CA).

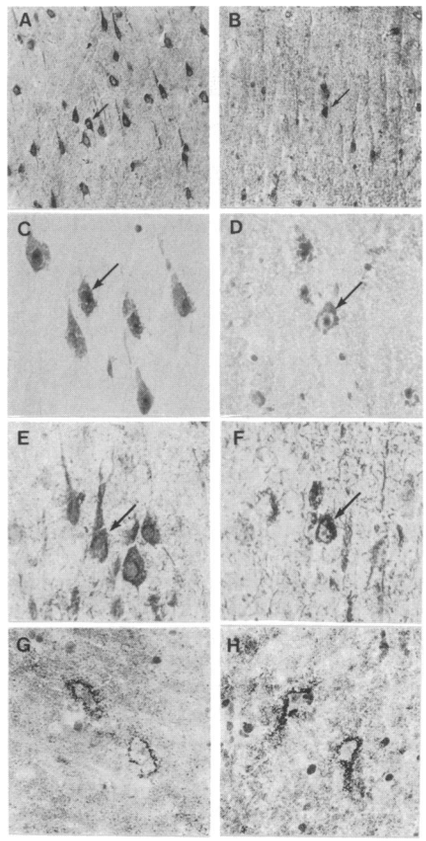

FIG. 1. Neuronal subset loss in AD subiculum: Pyramidal neurons of normal controls and AD patients. (*A* and *B*) mAb 3F12 (immunoperoxidase, ×55). (*C* and *D*) Nissl (×150). (*E* and *F*) mAb 44.1 (immunoperoxidase, ×150). (*G* and *H*) mAb 6A2 (immunoperoxidase, ×150). Serial sections of normal controls show cytoplasmic staining of pyramidal neurons by mAbs 3F12 and 44.1 (*A*, *C*, and *E*). The arrows indicate the same neuron in each serial section. There is staining of surface membranes of stellate neurons by mAb 6A2 (*G*). Companion serial sections of AD tissues show loss of most pyramidal neurons in Nissl-, mAb 3F12-, and mAb 44.1-stained sections (*B*, *D*, and *F*). An intact neuron is indicated in each section by the arrow. The stellate population in *G* and *H* (nonserial sections) is intact.

RESULTS

Hippocampus: Loss of Neuronal Subpopulation Identified by mAbs. Our studies indicate a selective loss of distinct neuronal subpopulations within the hippocampus and cerebral cortex in all nine AD patients. Within the hippocampal formation (Figs. 1*A* and 2*A*) of all normal and non-AD controls, antigen 3F12 is distributed in the soma, axons, and dendrites of most pyramidal neurons of CA1, subiculum, layers 3 and 4 of the entorhinal cortex, and rare neurons in CA2 to -4. In AD, there is loss of mAb 3F12-reactive material within these sites and a visible decrease in the number of labeled pyramidal neurons compared with adjacent Nissl-stained sections (Figs. 1 *A–D* and 2*B*). In contrast to the pattern of mAb 3F12, dentate granule neurons and pyramidal neurons in CA2 to -4 and in layer 6 of the entorhinal cortex remain essentially intact in Nissl-stained sections in AD and all control tissues.

In control tissues, antigen 44.1 is present in the perikaryal cytoplasm and axons of all cells in the above subregions (Figs. 1*E* and 2*A*), and in AD reactive material is also lost or decreased in these areas (Figs. 1*F* and 2*B*). The antibody also binds to, in control tissues, neurons of the stratum granulosum of the dentate gyrus, CA2 to -4, and neurons in layers 2, 4, and 6 of the entorhinal cortex (Fig. 2*A*). In AD, layer 2 cells of the entorhinal cortex show the major loss of mAb 44.1-binding neurons (Fig. 2*B*). mAb 6A2, which identifies nonpyramidal cells (stellate interneurons) in the subiculum and in layers 4 and 6 of entorhinal cortex, shows no loss of binding in AD or control tissues (Figs. 1 *G* and *H* and 2 *A* and *B*).

Neocortex: Neuronal Reactivity with mAbs. Within the neocortex of all AD patients, material reactive with mAb 3F12 is also markedly reduced or absent, but many neurons remain identifiable with mAbs 44.1 and 6A2. No neuronal changes were observed either in the normal or other neurologically diseased controls. The mAb 3F12-reactive neurons are a subset of those stained with mAb 44.1, but there are also intermingled neurons reactive only with mAb 44.1. It is, therefore, more difficult to identify neuron loss in these sites than in the hippocampal formation. Not all neurons that react with mAb 3F12 are affected by AD. Antigen 3F12 is present in a subpopulation of small neurons in the spinal cord anterior horns, the dorsal root ganglia, and the inner segments of retinal rod photoreceptor cells in control (9) and AD tissues (data not shown).

mAb 3A4: Neurofibrillary Tangle-Specific Binding. mAb 3A4-binding pattern within the hippocampal formation corresponded to the distribution of the neuronal loss in AD (Figs. 2 *B* and *C* and 3*F*). In some neurons, the antigen appeared as

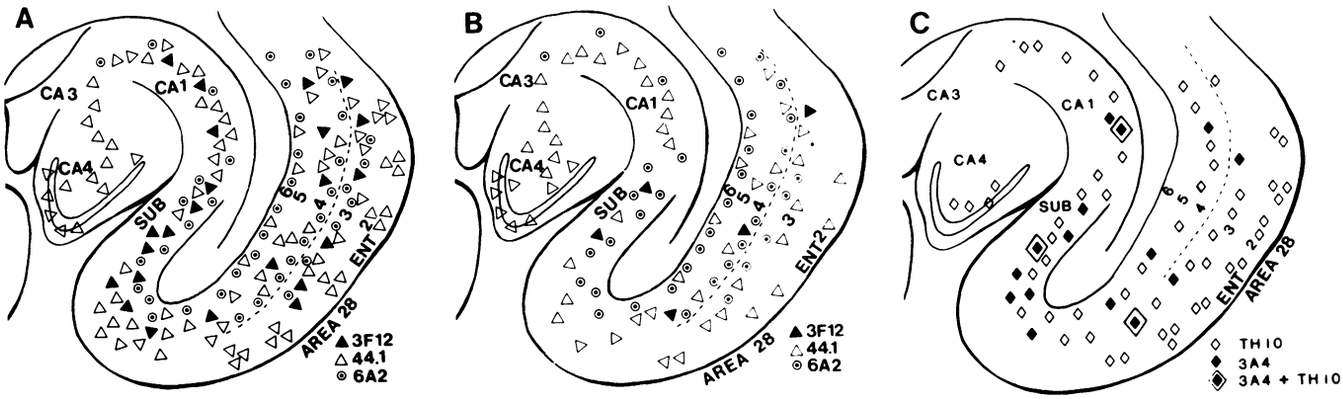

FIG. 2. Schematic diagram of mAb immunolocalization of neuronal subsets in hippocampal formation and entorhinal cortex. mAbs 3F12, 44.1, and 6A2 in control brains (*A*) and AD brains (*B*) (average duration, 8 years). In *C*, the distribution of mAb 3A4 reactivity is compared to thioflavin S staining. SUB, subiculum; ENT, entorhinal cortex. In AD note selective loss of neurons reactive with mAb 3F12; and in cells of layer 2 of the entorhinal cortex note loss of neurons reactive only with mAb 44.1. In *C* antigen 3A4 follows the distribution of neuronal loss.

multiple, small, discrete globular cytoplasmic aggregates (Fig. 3*B*) and in others as large elongate inclusions (Fig. 3*E*). Only scant mAb 3A4 binding was observed in the hippocampus of one of four age-matched normal controls. Reactivity was absent in all of the non-AD neurologically diseased controls, including the sites of primary neuronal degeneration: the caudate nucleus in patients with Huntington disease; anterior horn cells in spinal cords from amyotrophic lateral sclerosis patients, and cerebral cortex and hippocampus in patients with alcoholic dementia.

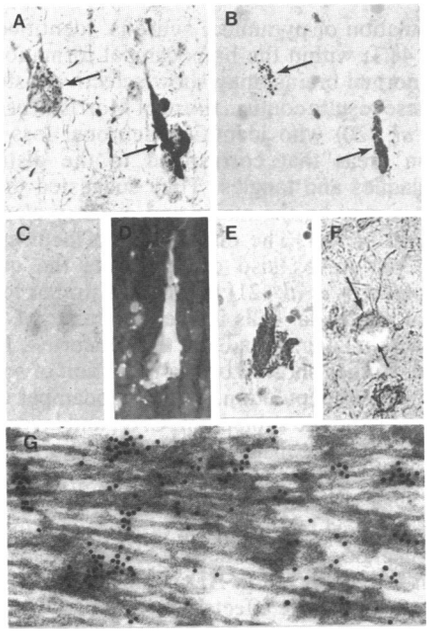

FIG. 3. Neurofibrillary tangle reactive material in pyramidal neurons in the subiculum. Serial sections of AD tissues containing neurons with neurofibrillary tangles and cytoplasmic globules (arrows) (*A* and *B*). (*A*) Bielschowsky stain (×225). (*B*) mAb 3A4 (immunoperoxidase) (×225). (*C*) Normal control tissues show no staining with mAb 3A4 (×85). Serial sections of AD tissue stained with thioflavin S (*D*) and with mAb 3A4 (*E*) show a cytoplasmic tangle reactive with both stains (×200). In *F* a pyramidal neuron in AD tissue is reactive with mAb 3F12 (long arrow; immunoperoxidase) and contains a tangle reactive with mAb 3A4 (short arrow; immunofluorescence) (×200). (*G*) Immunoelectron microscopy of stratum pyramidal in AD hippocampus reveals PHF within some pyramidal neurons labeled specifically with mAb 3A4 (immunogold, 10-nm particles). (×64,600.)

To determine the relationship of antigen 3A4 to neurofibrillary tangles, thioflavin S- and mAb 3A4-binding patterns were compared in the same or adjacent sections of AD hippocampus. The thioflavin S-, Congo red-, and anti-paired helical filament (PHF) mAb-binding patterns co-localized. The Bielschowsky reaction detected changes in cells also recognized with either thioflavin S or mAb 3A4. Some neurons stained only with mAb 3A4, and others stained solely with thioflavin S. A third, minor group of neurons had cytoplasmic material reactive with mAb 3A4 and with thioflavin S (Fig. 2*C*). Ultrastructurally, immunogold binding (Fig. 3*G*) was localized to PHF in some neurons.

Topography of mAb-Binding Sites and Duration of Disease. The distribution of antigen 3A4- and thioflavin S-reactive tangles may be related to the duration of the disease. The length of the clinical course, dated from the time the patient first became symptomatic, was determined from a careful clinical history obtained from the caregiver and further verified by repeated neuropsychological testing. As seen in Table 1, antigen 3A4, essentially absent in normal brains, became predominant in brains from patients with a short clinical course. Clinical duration of a decade or longer was accompanied by a decrease or absence of mAb 3A4-binding material in all hippocampal sites. Age-matched normal and non-AD neurologically diseased controls failed to show significant mAb 3A4 binding or mAb 3F12 cell-specific loss in the hippocampus and other sites affected in AD. Although we have surveyed only a limited number of patients, our studies suggest that layer 2 of the entorhinal cortex may be the earliest and most severely affected area, followed by the subiculum, CA1 of hippocampus, deeper layers of entorhinal cortex, association areas of neocortex and, lastly, primary sensory cortex. In patients 1 and 2, where the patient's demise occurred 1–3 years after diagnosis, affected neurons in layer 2 of the entorhinal cortex contained material that reacted extensively with mAb 3A4 as well as thioflavin S. In all other cases, layer 2 neurons were filled only with thioflavin S-reactive material. Conversely, in the case of longest clinical duration (patient 9), there were abundant neurons with thioflavin S-reactive material in the hippocampus, and mAb 3A4-reactive material was present in association cortex and primary neocortex (area 17). These results suggest that antigen 3A4 appears in neurons prior to thioflavin S-reactive material.

Immunoblots: Antigens Identified by mAbs. Antigens identified by mAbs 3F12 and 3A4 were analyzed on immunoblots using hippocampal or neocortical homogenates. mAb 3F12 identified a major 49-kDa band and minor bands of 60, 45, and 42 kDa in homogenates of normal and AD tissues (Fig. 4,

Table 1. Neurofibrillary tangles in subiculum of AD patients with various durations of disease

Patient	Age, yr	Duration of disease, yr	Tangles stained, %		
			Thio	3A4	Thio/3A4
1	88	1	28	72	12
2	76	3	45	55	22
3	77	4	43	57	21
4	94	5	56	44	32
5	72	6	48	52	22
6	89	8	62	38	16
7	87	10	85	15	6
8	90	11	93	7	4
9	78	16	100	0	0

Tangles identified by mAb 3A4, by thioflavin S, or by both were correlated with the duration of disease as timed from the onset of clinical symptoms to the patient's demise. Total tangles are defined by the sum of tangles reactive with either mAb 3A4 or thioflavin minus those stained by both. A minimum of 100 tangles per patient were counted. Single staining by either thioflavin S (Thio) or by mAb 3A4 (immunoperoxidase) or double staining by both methods (Thio/3A4) was on serial sections. Landmarks common to both sections (e.g., blood vessels) were chosen at random as reference points. The superimposition of landmarks on adjacent photomicrographs allowed the number and type of tangles to be counted in specific neurons. Coefficient of correlation of antigen 3A4 to duration of disease: $r = -0.96$. Note the higher percentage of neurons containing antigen 3A4 than thioflavin S-reactive material in tissues of patients dying within 5 years after initial onset of symptoms. With time, a reduction of mAb 3A4-reactive tangles is seen; by 16 years there is no mAb 3A4-binding material in the subiculum, and all tangles stain only with thioflavin S.

lanes b and c). Although the 60-kDa band was somewhat variable in intensity among homogenates from controls, from two AD patients (patients 8 and 9) with clinical disease of long duration, loss of mAb 3F12 binding to the 60-kDa band may be related to the marked decrease of mAb 3F12-reactive material seen in neurons in immunocytochemically stained sections. No protein band was sensitive to incubation with alkaline phosphatase. In contrast to the neurospecificity of antigen 3A4, antigen 3F12 was immunocytochemically detected in nonneural tissues, including squamous epithelium, lymph nodes, and the medium of cultured small blood vessels.

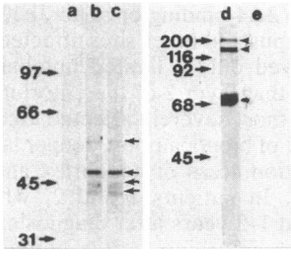

FIG. 4. Immunoblots of hippocampal homogenates. Unfixed, frozen hippocampal tissues were homogenized, electrophoresed, and electroblotted onto nitrocellulose. Blots were immunolabeled with ^{125}I-labeled goat anti-mouse IgG/IgM mixture (2 μCi/μg; 1 Ci = 37 GBq), dried, and visualized by autoradiography. Lanes: a, normal tissue homogenate with no primary mAb; b, mAb 3F12 staining of normal tissue homogenate; c, mAb 3F12 staining of AD tissue homogenate [note reactive bands of 60 (small-arrow), 49, 45, and 42 kDa]; d, electrophoretic separation of purified (15, 16) neurofilament triplet proteins (9 μg per sample) from gray matter electrophoresed on 10% gels and stained with Coomassie blue (200, 150, and 70 kDa, arrowheads); e, immunoblot of the same gel stained with mAb 3A4. No reactive material in any component of a neurofilament preparation was detected. Large arrows indicate molecular weight standards.

Although mAb 3A4 binds to some component of PHF, molecular characterization of antigen 3A4 has been more elusive. Whereas mAb 3A4 is nonreactive in the normal human CNS, it reacts strongly with the normal human retinal photoreceptor outer segment and with axons in the *Drosophila* nervous system but faintly with neuronal nuclei in both species (9). In all human AD and control tissues and in *Drosophila* heads, mAb 3A4 binding was inactivated when the tissue was treated with alkaline phosphatase. Antigen 3A4 was insoluble in 2.3% (wt/vol) $NaDodSO_4$ but still immunohistochemically reactive, and immunoblots of tissue homogenates of normal or AD human brain or *Drosophila* heads were nonreactive with mAb 3A4. These findings suggest that mAb 3A4 may be binding to a phosphorylated, highly insoluble protein. Immunocytochemical tests with antibodies to lamin, a neurofilament-like nuclear protein, to the microtubule-associated protein tau (17), and to the Alz-50 antigen (18) did not cross react with the mAb 3A4-positive cytoplasmic material in AD tissues. mAb 3A4 failed to react with purified neurofilament protein from normal human cerebral cortex or white matter. The purified 200-, 150-, or 68-kDa triplet proteins did not bind mAb 3A4 in immunoblots (Fig. 4, lane b) or to dot blots (data not shown) but did bind mAbs with known specificities for phosphorylated and nonphosphorylated neurofilament proteins (data not shown). Purified preparations of bovine tau and bovine and rabbit microtubule proteins did not bind mAb 3A4, whereas anti-tau mAb (17) and mAb Alz-50 (18) gave similar immunoblot patterns. In preliminary experiments, incubation of mAb 3A4 supernatants with purified tau, neurofilament proteins, or ubiquitin failed to alter its binding pattern on tissues (data not shown).

DISCUSSION

A subpopulation of pyramidal neurons, identified by mAbs 3F12 and 44.1, within the hippocampal formation and neocortex in normal brains, may show selective histopathology in AD. These results confirm those of Morrison *et al.* (19) and Lewis *et al.* (20) who identified neuronal loss in cortical association areas that correspond to the distribution of neuritic plaques and tangles. They suggested that intrinsic cortical loss may be the major and primary element in the pathogenesis of AD. The distribution of neuronal loss detected by our mAbs also complements the quantimetric analyses of Terry *et al.* (21) that demonstrated loss of some neocortical pyramidal cells in the size range of >80 μm in diameter within layers 3 and 5 of the neocortex. In the aging brain those studies showed only a reduction of somal size in that neuronal subpopulation. The hippocampal distribution of neuronal loss in the subiculum, CA1, and layers 2–4 of the entorhinal cortex is consistent with that reported by Hyman *et al.* (22). The early loss of layer 2 neurons of the entorhinal cortex, a way-station for corticohippocampal projections, may be the critical factor in loss of memory function.

The cholinergic system is the best recognized neurotransmitter system to be affected in AD, although not all cholinergic neuronal cell groups are affected. Intrinsic cortical cholinergic neurons have been described in the rat brain, but, in contrast to neurons identified by mAb 3F12, they are nonpyramidal in shape and are mainly in layers 2 and 3 (23). Glutamate, a likely candidate for the neurotransmitter of these intrinsic pyramidal neurons, is also affected in AD. There is a loss of glutamate in the molecular layer of the dentate gyrus, the site of perforant pathway terminals (18) and of glutamate uptake in cortical and hippocampal regions (24).

We postulate that, at the cellular level, there is a temporally related pattern of immunocytochemically detectable changes occurring in pyramidal neurons affected by AD (Fig. 5). The first normally present antigen to disappear or to become nonreactive is antigen 3F12. Antigen 44.1, also initially present, later becomes undetectable. Antigen 3A4 may be an

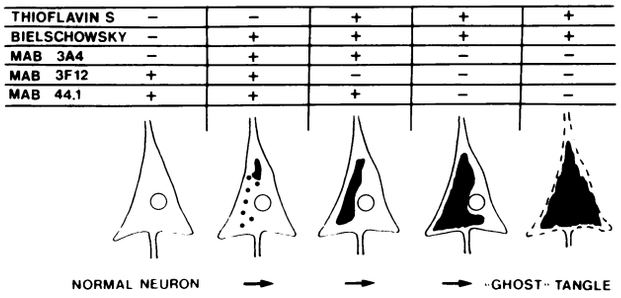

FIG. 5. Hypothetical scheme of antigen loss and progression of tangle formation within an AD pyramidal neuron. Stains are as indicated. +, Reactive; −, nonreactive. Dots are intracytoplasmic globules; the large masses represent tangles. Dotted line surrounding ghost tangle indicates that the plasma membrane is no longer visible.

earlier indicator of AD than other markers, including mAbs prepared with isolated tangles as immunogen. This antigen becomes detectable prior to loss of cellular reactivity with mAb 3F12, mAb 44.1, or the thioflavin S stain. With time, the thioflavin S-reactive material remains and is more abundant than mAb 3A4-reactive material. After a long clinical course, mAb 3A4-reactive material may be detectable only in the neocortex, including primary areas. From these studies, it cannot be determined if antigen 3A4 is no longer present in the tissues or is further modified and no longer detectable.

Predictably, young, normal controls showed none of the AD-specific changes. Because of the paucity of autopsy tissues available from neurologically evaluated normal, age-matched controls, it is more difficult to assess histologic changes, especially within the hippocampal formation, where some histopathology of AD may be seen in the absence of dementia. Whether these cases are incipient AD or merely age-related changes is not clear. Evaluation of other, rarer dementing disorders such as Pick disease is needed but must be prospectively planned.

Direct biochemical characterization of antigen 3A4 has not yet been achieved because of its insolubility under standard conditions. The mAb appears to recognize a phosphorylated epitope, since treatment with phosphatase eliminates its reactivity. Neurofilament proteins, microtubule-associated protein 2 (25), and tau proteins (26) have been demonstrated to be abnormally phosphorylated in AD. The absence of mAb 3A4 binding to neurofilaments isolated from human white or gray matter after $NaDodSO_4$ treatment does not exclude the possibility that antigen 3A4 is of neurofilament origin and becomes incorporated into PHF. Native antigen 3A4 may be in a modified form possibly linked to a nonprotein portion of the molecule or present in quantities not detectable under gel electrophoresis conditions.

Similarly, the neuron-specific protein recognized by mAb 3F12 may be the 60-kDa species noted on immunoblots. Its lability in our preparations has so far made direct characterization difficult. Use of the antibody to screen a cDNA expression library could lead to further insight into the role of this protein and its corresponding gene in subset-specific function. Our observations may be useful in defining cellular mechanisms, and determining the role of genetically determined factors in the neurons specifically vulnerable to degeneration in Alzheimer disease.

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